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Intramuscular Vascular Endothelial Growth Factor Gene Therapy in Patients with Chronic Critical Leg Ischemia

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PURPOSE: We sought to investigate the safety and efficacy of intramuscular gene therapy with vascular endothelial growth factor (VEGF) in patients with chronic critical leg ischemia.

METHODS: Gene transfer was performed in 24 limbs of 21 patients with rest pain, some of whom also had nonhealing ischemic ulcers (n = 16) due to occlusive peripheral arterial disease. Between 400 μg and 2000 μg of phVEGF$_{165}$ (400 μg, n = 2; 800 μg, n = 4; 1200 μg, n = 4; 1600 μg, n = 6; and 2000 μg, n = 8) was injected directly into the muscles of the ischemic limb; the same dose was injected 4 weeks later. The ratio of blood pressures at the ankle and brachial artery was measured before and after treatment.

RESULTS: Mean (± SD) plasma levels of VEGF increased significantly from 26 ± 31 pg/mL to 63 ± 56 pg/mL (P <0.005), and the ankle-brachial index improved significantly from 0.58 ± 0.24 to 0.72 ± 0.28 (P <0.001). Magnetic resonance angiography showed qualitative evidence of improved distal flow in 19 limbs (79%). Ischemic ulcers healed or improved markedly in 12 limbs (75%). Rest pain was relieved or improved markedly in 20 limbs (83%). Amputation was performed in two limbs because of wound infection. Complications were limited to transient leg edema in six limbs.

CONCLUSION: Intramuscular gene therapy with VEGF$_{165}$ for patients with chronic critical leg ischemia is safe, feasible, and effective. **Am J Med. 2003;114:85–92.** ©2003 by Excerpta Medica Inc.

Despite advances in the treatment of occlusive peripheral arterial disease, many patients cannot be managed adequately with either medical therapy (1) or revascularization procedures (2). In some patients, the anatomic extent and distribution of arterial occlusion are too severe to permit relief of pain or healing of ischemic ulcers. Therapeutic angiogenesis, by promoting development of collateral vessels, may be useful in these patients (3,4).

Several growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor, can stimulate the development of collateral vessels in animal models of hind limb ischemia (5,6). Among the growth factors, VEGF is specifically mitogenic for endothelial cells (7–9), an important advantage of VEGF for gene therapy because endothelial cells are responsible for neovascularization. Intramuscular gene therapy with VEGF promotes collateral vessel development, relieves ischemic symptoms, and improves endothelial function in patients with critical limb ischemia (10,11). However, the minimal effective dose of VEGF plasmid is uncertain, and it is also not known whether racial differences affect therapeutic results. Therefore, we studied several doses of intramuscular VEGF plasmid for the treatment of chronic critical leg ischemia in Chinese patients.

METHODS

Patient Selection

Patients qualified for intramuscular gene therapy if they had chronic critical limb ischemia (12), including rest pain or nonhealing ischemic ulcers for a minimum of 4 weeks without evidence of improvement in response to conventional therapies, and were not optimal candidates for surgical or percutaneous revascularization (13). The ratio of systolic blood pressure at the ankle and the brachial artery (the ankle-brachial index) was <0.6, or the great toe pressure was <30 mm Hg in patients with noncompressible ankle arteries. All patients had angiographic evidence of superficial femoral artery or infrapopliteal disease in the index limb. All patients had no new medications started and did not stop any medications that would have reduced flow. We excluded patients with radiographic and radioisotopic evidence of osteomyelitis in the ischemic extremity; a history of alcohol or drug abuse within the past 3 months; previous or current history of

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neoplasm; hepatic dysfunction; or evidence of possible malignancies following evaluation with carcinoembryonic antigen levels, chest radiographs, computerized tomographic scan, and mammography in women or prostate examination and prostate-specific antigen levels in men. The protocol was approved by the Human Research Committee of our institution and the Department of Health, Executive Yuan, Taiwan. All patients gave written informed consent to participate.

Patients were followed on a biweekly basis for the first 10 weeks after gene therapy, and at monthly intervals thereafter. Ischemic ulcers were documented by color photography. Shrinkage of the ulcer area to less than half of the baseline area was defined as improvement. Improvement in rest pain was evaluated by questionnaire and the amounts of analgesic medications used before and after therapy. Visual acuity and fundoscopic examination were checked before and 4 weeks after the second injection of plasmid in patients with diabetes mellitus.

Plasmid Deoxyribonucleic Acid ($phVEGF_{165}$) Preparation and Administration

All patients received a eukaryotic expression vector encoding the $VEGF_{165}$ gene (14,15). Preparation and purification of the plasmid from cultures of $phVEGF_{165}$-transformed *Escherichia coli* were performed in the central laboratory at our hospital with the endotoxin-free column method (Qiagen Mega Kit, Qiagen Inc., Valencia, California). The purified plasmid was stored in vials and pooled for quality-control analysis.

Aliquots of 400 μg of $phVEGF_{165}$ were diluted in sterile saline, and one to five 2-mL aliquots (total, 400 μg to 2000 μg) were injected directly into the calf muscles of the ischemic limb. The injection sites were selected arbitrarily according to available muscle mass in the calf. A second injection of the same dose was administered 4 weeks later. Patients were not aware of the dose they received.

Plasma Levels of Vascular Endothelial Growth Factor

Plasma VEGF levels were measured at baseline and 2 weeks after the first dose of VEGF to detect evidence of gene expression. Samples were immediately centrifuged for 20 minutes at 4000 rpm at 4°C and stored at –70°C until analysis. Plasma VEGF levels were measured with an enzyme immunoassay technique according to the manufacturer's instruction (R & D Systems, Inc., Minneapolis, Minnesota). Results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/mL. Samples were checked by serial dilution and were performed at least in duplicate. The intra-assay coefficient of variation was 4.1%, and the interassay coefficient of variation was 5.6%.

Ankle-Brachial Index

Resting ankle-brachial indices were calculated as the ratio of the lowest pressure from either the posterior or anterior tibial arteries divided by the greatest brachial systolic pressure, which were obtained 1 week before and 4 weeks after completing the two injections. A technician who was unaware of the treatment status of the patients performed this examination.

Magnetic Resonance Angiography

Moving-bed infusion-tracking magnetic resonance (MR) angiography (16) was performed 1 week before and 4 weeks after completing the two injections. Angiograms were obtained with a 1.5-T MR system (Impact; Siemens, Erlangen, Germany). A body coil was used for signal transmission and reception. The dynamic study was acquired twice; before infusion of contrast material and during infusion. To obtain accurate and reproducible table movement, a wooden stick with three premeasured stops was used. After acquisition of the unenhanced study, 0.4 mL/kg of preheated gadopentetate dimeglumine was injected. The two dynamic studies were reconstructed; nonenhanced volumes were subtracted from corresponding gadolinium-enhanced volumes in all regions. An increase in the number of visible vessels, or an increase in the intensity or apparent size of a previously visible vessel, was considered improvement. A radiologist who was not aware of the treatment status of the patients interpreted the MR angiograms. Quantitative MR angiographic analysis of collateral vessel development was used to derive an angiographic score for each film, defined as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections from knee to ankle.

Statistical Analysis

Paired *t* tests were used to compare continuous variables before and after therapy; analysis of variance followed by Scheffé's procedure was used to compare three or more means. A value of $P < 0.05$ was considered significant.

RESULTS

A total of 21 patients with 24 limbs met all eligibility criteria and were treated with intramuscular injections of $phVEGF_{165}$. Their mean ($\pm$ SD) age was 65 $\pm$ 16 years (range, 21 to 84 years), and 6 of the patients were women (Table). Patients had rest pain in all 24 limbs; there were nonhealing ischemic ulcers in 16 limbs and gangrene in 4 limbs. Five patients had a prior surgical bypass graft, and 4 had undergone previous toe amputations. Six patients were current smokers, and 6 were ex-smokers; 10 had diabetes mellitus, 7 had hypertension, and 6 had hyperlipidemia.

Table. Clinical Characteristics of Patients, Dose Protocol for phVEGF$_{165}$, and Leg Edema after Therapy

Patient No.	Sex	Age (years)	Smoking	Diabetes Mellitus	Signs and Symptoms	Affected Limb*	Underlying Vascular Disease	VEGF Dose	Booster (2000 μg)	Edema after Therapy
1	M	72	+	+	Rest pain	Left	Atherosclerosis	400 μg × 2	+	0
2	F	67	0	+	Rest pain	Left	Atherosclerosis	400 μg × 2	+	0
3	M	84	+	0	Rest pain	Left	Atherosclerosis	800 μg × 2	0	0
					Rest pain	Right	Atherosclerosis	1200 μg × 2	0	0
4	M	75	+	+	Rest pain	Left	Atherosclerosis	800 μg × 2	+	0
5	F	75	0	+	Rest pain, ulcer	Left	Atherosclerosis	800 μg × 2	0	0
6	M	39	+	0	Rest pain	Right	Burger's disease	800 μg × 2	0	0
					Rest pain	Left	Burger's disease	2000 μg × 2	0	0
7	M	70	0	+	Rest pain	Left	Atherosclerosis	1200 μg × 2	0	0
8	M	74	0	+	Rest pain, ulcer	Left	Atherosclerosis	1200 μg × 2	0	0
9	M	41	+	0	Rest pain, ulcer	Left	Burger's disease	1200 μg × 2	+	0
10	M	73	+	+	Rest pain, ulcer	Left	Atherosclerosis	1600 μg × 2	0	0
					Rest pain, ulcer	Right	Atherosclerosis	2000 μg × 2	0	+
11†	M	61	0	+	Rest pain, ulcer	Right	Athersclerosis	1600 μg × 2	0	0
12	M	53	0	0	Rest pain, ulcer	Left	Atherosclerosis	1600 μg × 2	+	+
13	M	21	+	0	Rest pain, ulcer	Right	Burger's disease	1600 μg × 2	0	+
14	M	76	+	0	Rest pain, ulcer, toe gangrene	Right	Atherosclerosis	1600 μg × 2	0	+
15	F	59	0	+	Rest pain, ulcer	Right	Atherosclerosis	1600 μg × 2	0	+
16	F	71	+	0	Rest pain, ulcer	Left	Atherosclerosis	2000 μg × 2	0	0
17†	M	78	0	+	Rest pain, ulcer, toe gangrene	Left	Atherosclerosis	2000 μg × 2	0	0
18†	F	78	0	0	Rest pain, ulcer	Right	Atherosclerosis	2000 μg × 2	0	+
19	M	73	+	0	Rest pain, ulcer, toe gangrene	Right	Atherosclerosis	2000 μg × 2	0	0
20	M	42	+	0	Rest pain, ulcer	Left	Burger's disease	2000 μg × 2	0	0
21	F	79	+	+	Rest pain, ulcer, toe gangrene	Left	Athersclerosis	2000 μg × 2	0	0

+ = present; 0 = not present; F = female; M = male; VEGF = vascular endothelial growth factor.

* Indicates the first leg that was treated. Patients 3, 6, and 10 were also treated in the contralateral leg.

† Amputation below the knee joint was performed in patients 17 and 18. Patient 11 also failed to respond to treatment.

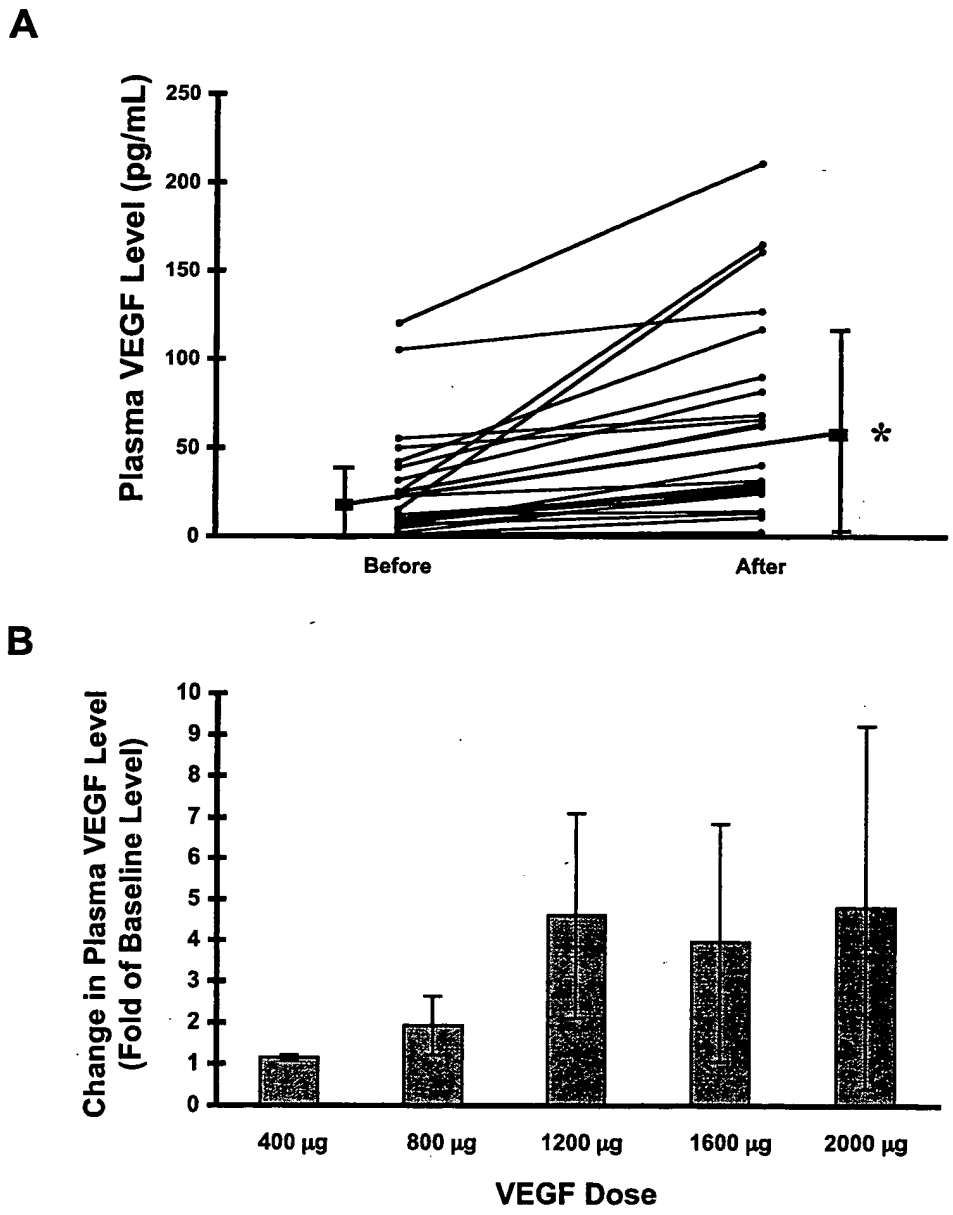

Figure 1. A: Plasma vascular endothelial growth factor (VEGF) levels before treatment and 2 weeks after intramuscular injection of phVEGF165. The dot and bar indicate mean ± SD; the asterisk indicates $P = 0.001$. B: Change in plasma VEGF levels by doses of phVEGF165. The bar indicates the mean; the error bar indicates the SD.

phVEGF$_{165}$ Dose

The dose of VEGF ranged from 400 µg to 2000 µg (Table). Several patients received a 2000-µg booster dose 2 to 3 months after the second injection (2 patients treated 400 µg, 1 patient treated with 800 µg, 1 patient treated with 1200 µg, and 1 patient treated with 1600 µg). Three patients had bilateral critical limb ischemia; the second limb was treated 1 month after treatment of the first limb. The maximal treatment dose for one limb was 5200 µg (patient 12); for bilateral treatment, the maximal dose was 7200 µg (patient 10).

Transgene Expression

Mean blood levels of VEGF increased significantly from 26 ± 31 pg/mL before treatment to 63 ± 56 pg/mL ($P < 0.005$) 2 weeks after the first dose of gene therapy (Figure 1A). The increase in VEGF levels, however, was highly variable; although there appeared to be a dose-related response (Figure 1B), it was not statistically significant ($P = 0.46$).

Clinical Follow-up and Safety Assessment

Intramuscular gene transfer induced no or mild local discomfort for up to 48 hours after injection. Mild (limited

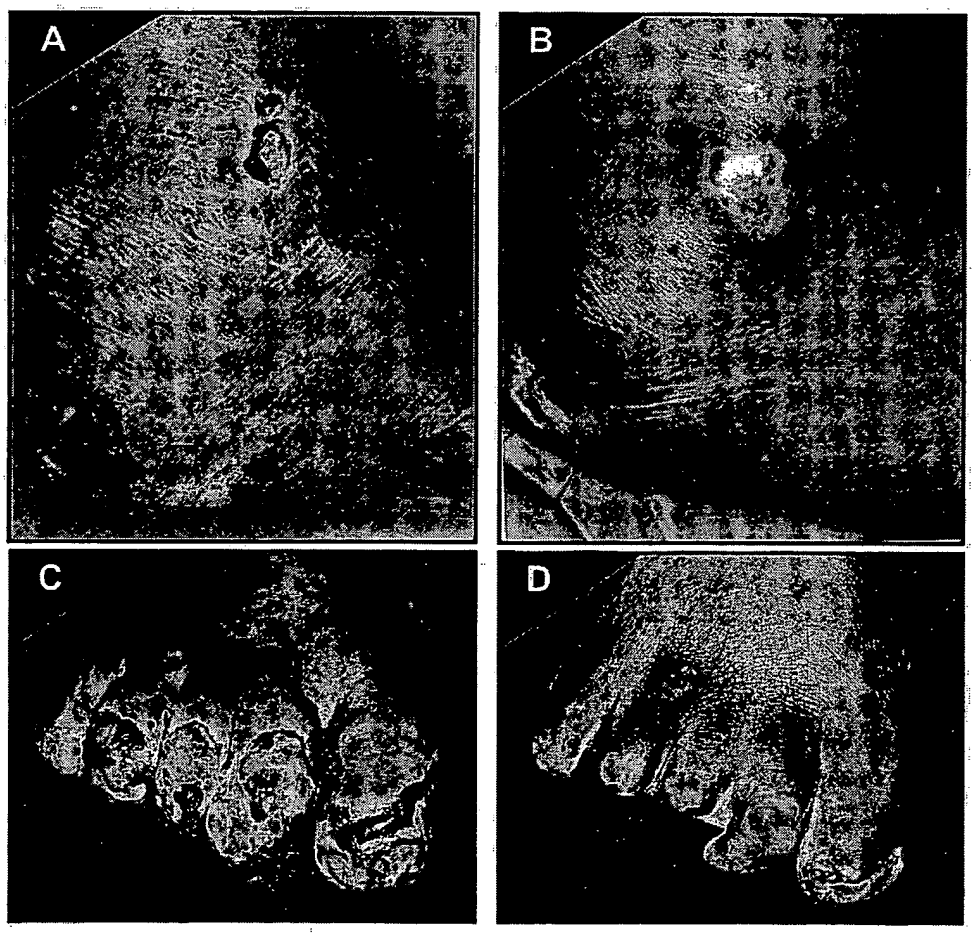

Figure 2. Limb salvage after gene therapy. A 76-year-old man presented with nonhealing wounds for 1 month on the right ankle and foot (A). After gene therapy (B), the wound healed completely. His ankle-brachial index increased by 0.37 in association with increased flow on magnetic resonance angiography. A 39-year-old man presented with nonhealing ulcers on the right foot for 1 year (C). After gene therapy (D), the wounds improved markedly. The ankle-brachial index increased by 0.2 after gene therapy.

to below the ankle) and transient leg edema occurred in 6 (25%) of the 24 treated limbs, and only in patients with nonhealing ischemic ulcers who had been treated with 1600 or 2000 μg of VEGF (Table). Edema subsided spontaneously without use of diuretics.

All patients were followed for at least 6 months. Amputation of the affected limb because of a large ulcer with a severe wound infection was performed in 2 patients who had been treated with 2000 μg of VEGF. Rest pain or the leg ulcer improved markedly within 1 month in 4 of the 5 patients treated with a booster injection for persistent rest pain.

Overall, rest pain resolved completely in 12 limbs and improved markedly in eight limbs. The ischemic ulcer healed in six limbs (Figure 2) and improved markedly in 6 limbs. Thus, therapeutic benefit was demonstrated by regression of rest pain in 20 (83%) of 24 ischemic limbs and improved tissue integrity in 12 (75%) of 16 ischemic limbs with ulceration. Treatment failure occurred in 2 patients treated with 400 μg, 1 patient treated with 800 μg, 1 patient treated with 1200 μg, and 1 patient treated with 1600 μg. No diabetic patient had a decline in visual acuity or clinically apparent fundoscopic changes.

Change in Ankle-Brachial Index

The mean ankle-brachial index increased significantly from 0.58 ± 0.24 before treatment to 0.72 ± 0.28 ($P < 0.001$) 4 weeks after completing two injections (Figure 3A). The increase in ankle-brachial index in patients receiving 1600 μg was significantly higher than that in patients receiving 400 μg or 800 μg (Figure 3B), although there was no further improvement in patients who received the 2000-μg dose. The mean ankle-brachial index in the 5 patients who received booster doses changed from 0.47 ± 0.22 before therapy to 0.56 ± 0.21 after two injections to 0.66 ± 0.18 after the booster dose.

Change in Magnetic Resonance Angiography

Magnetic resonance angiography showed superficial femoral artery or infrapopliteal disease in all 24 limbs, 19 of which showed qualitative evidence of improved distal flow after gene therapy (Figure 4). The mean MR angio-

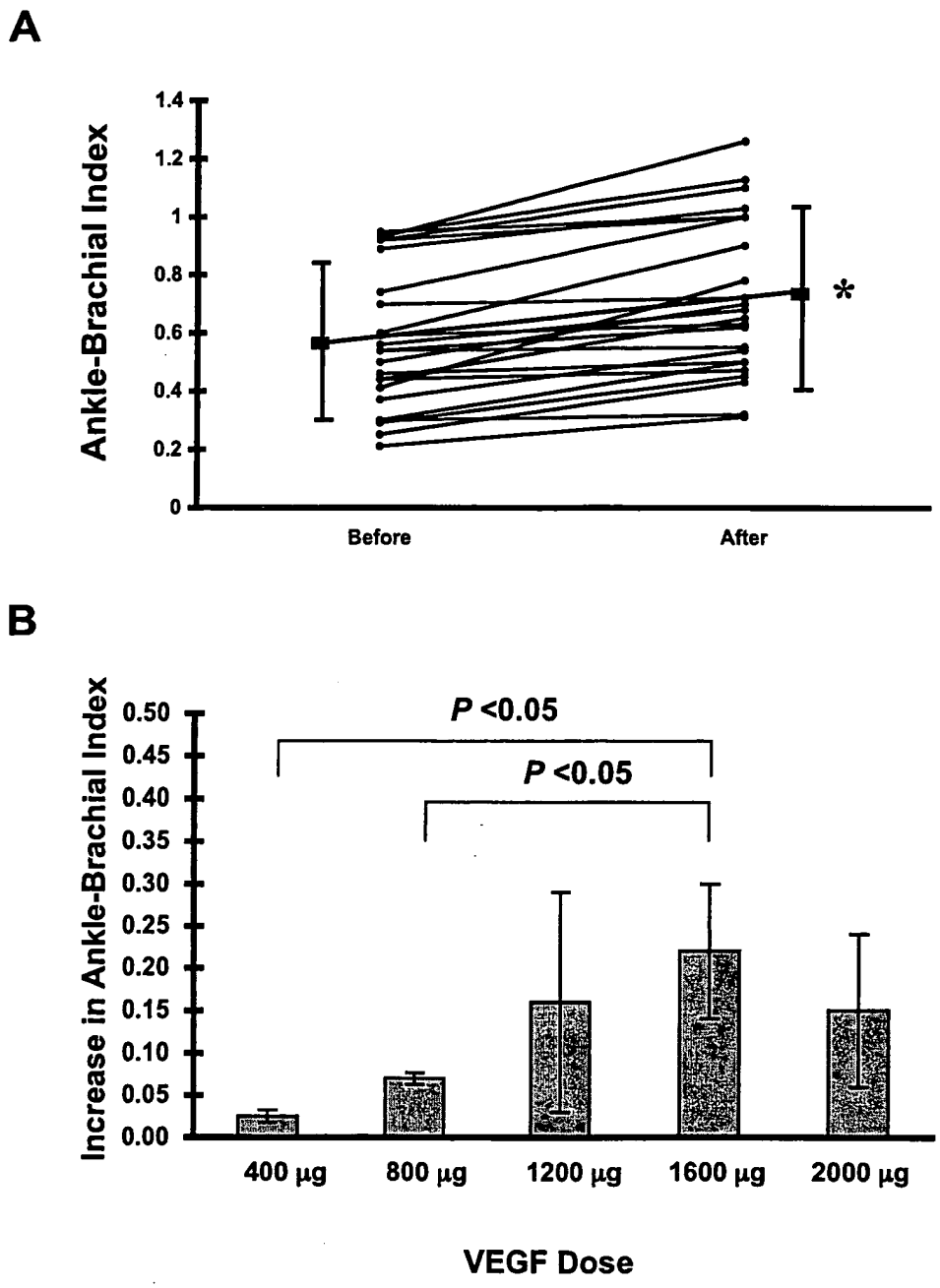

Figure 3. A: Ankle-brachial index before and 4 weeks after gene therapy. The dot and bar indicate mean ± SD; the asterisk indicates $P < 0.001$. B: Increase (mean ± SD) in ankle-brachial index by doses of phVEGF165. VEGF = vascular endothelial growth factor.

graphic score increased significantly from 0.37 ± 0.10 before treatment to 0.47 ± 0.11 after gene therapy ($P < 0.01$; (Figure 5).

DISCUSSION

The prognosis and quality of life for patients with chronic critical leg ischemia, as manifested by rest pain or ischemic ulcers, are poor (17), and no pharmacologic treatment has been shown to be effective in these patients (18). Indeed, amputation, despite the associated morbidity, mortality, and functional implications (19–21), is often recommended as a solution for disabling symptoms (22–25). Thus, the need for alternative treatment strategies is compelling. Intramuscular injection of naked plasmid deoxyribonucleic acid has a wide range of applications, because striated muscle can take up and express foreign genes (26–28). Gene therapy with VEGF has been used successfully in a patient with critical leg ischemia who was treated with arterial infusion of $VEGF_{165}$ plasmid (15). A

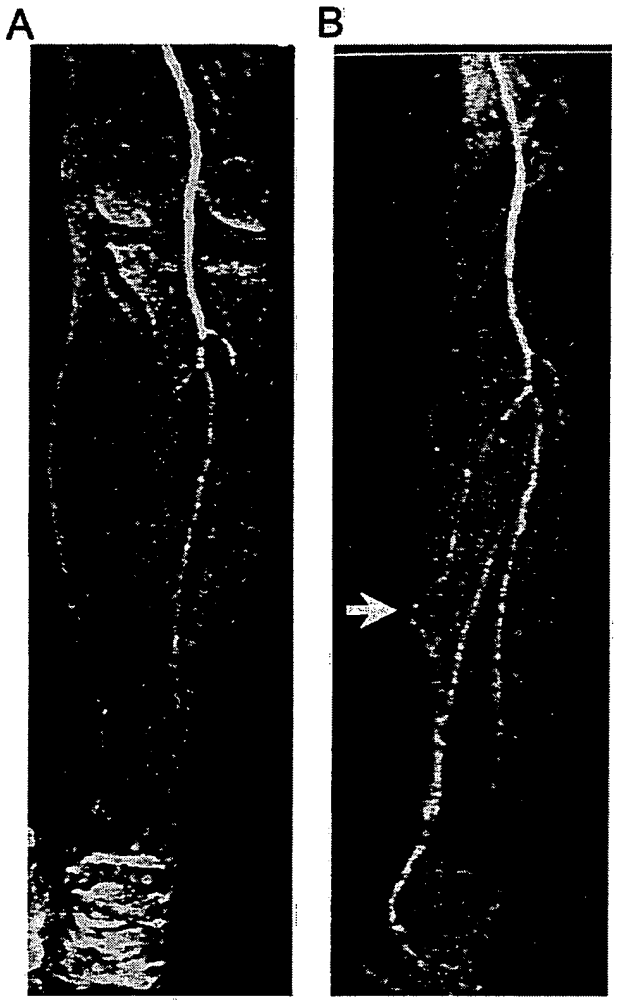

Figure 4. Magnetic resonance angiogram before (A) and 4 weeks after (B) gene therapy. After gene therapy, signal enhancement and collaterals are evident (arrow), consistent with improved flow in the ischemic limb.

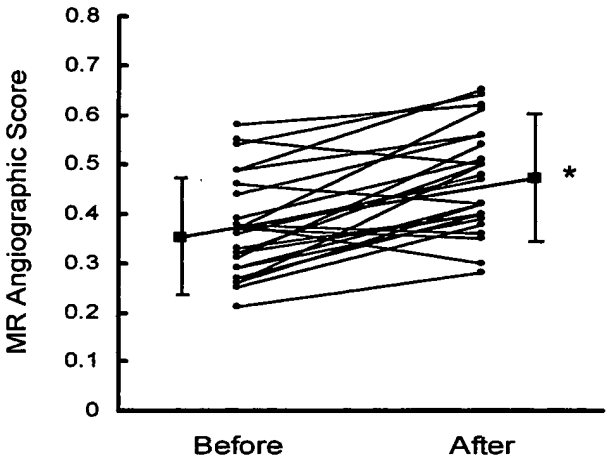

Figure 5. Magnetic resonance (MR) angiographic score before and 4 weeks after gene therapy. The dot and bar indicate mean ± SD; the asterisk indicates $P = 0.00$.

previous study showed that a fixed dose of intramuscular gene therapy with phVEGF$_{165}$ was safe and effective in the treatment of patients with critical limb ischemia (10). However, high-dose VEGF induces angioma formation in the rat heart (29), and the efficacy and safety of this kind of gene therapy needs clarification (30,31). Compared with that previous study (10), we observed a lower complication rate (25% vs. 67%) and a higher failure rate (25% vs. 10%), perhaps because we used a lower dose of phVEGF$_{165}$.

Although the ankle-brachial index, which is subject to measurement error, does not parallel local perfusion changes, an increase of >0.1 in the index indicates a successful surgical or percutaneous intervention (32). We observed increases in the mean ankle-brachial index after gene therapy in patients treated with at least 1200-μg injections of phVEGF$_{165}$, suggesting that 2400 μg of phVEGF$_{165}$ (all patients received two doses) is the minimal effective dose for the treatment of chronic critical leg edema in Chinese patients.

Traditionally, conventional arteriography has served as the gold standard for the diagnostic evaluation of lower extremity vascular disease. However, conventional arteriography is invasive and potentially hazardous. Magnetic resonance angiography has been shown to provide similar information as conventional angiography (16,33); it is noninvasive and can be performed in outpatients, as in our study.

Our study has several limitations. It was not randomized, placebo-controlled, or double-blind. However, we believe that the improvement in clinical symptoms and signs in our study cannot be attributed to a placebo effect, as patients were unaware of their phVEGF$_{165}$ dose, and there was no therapeutic benefit from low doses of phVEGF$_{165}$. Although patients who received higher doses appeared to have greater improvement, this finding cannot substitute for a true control group. We did perform the ankle/brachial index and MR angiographic examination in a blinded manner. Finally, we did not perform exercise testing to determine walking capacity.

In conclusion, we found that intramuscular VEGF gene transfer was safe and effective in Chinese patients with chronic critical leg ischemia. The benefits of treatment on the ankle-brachial index appeared to require at least a 1200-μg dose. Edema after intramuscular gene therapy with VEGF was dose related, perhaps because more patients with ulcers, who had a greater risk of edema, were treated with higher doses. Clinical efficacy, including resolution of rest pain, limb salvage, and healing of ischemic ulcers, was associated with objective findings of improved ankle-brachial indices and blood flow

on MR angiography. However, two limbs were amputated because of severe infection of an ulcer, emphasizing that meticulous wound care is necessary.

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